Summary of ACMG Classification Rules Specified for *PTEN* Variant Curation

Version 1.0, approved by the ClinGen Sequence Variant Interpretation Working Group on August 17, 2018

PATHOGENIC CRITERIA		
Criteria	**Criteria Description**	**Specification**
VERY STRONG CRITERIA		
PVS1	Null variant (nonsense, frameshift, canonical ±1 or 2 splice sites, initiation codon, single or multi-exon deletion) predicted to result in nonsense-mediated decay or causing truncation/frameshift at or 5' to c.1121 (NM_000314.6).	Disease-specific
PS2 or PM6_Very Strong	Two proven OR four assumed OR one proven + two assumed *de novo* observations in a patient with the disease and no family history.	Strength
PS4_Very Strong	Probands with specificity score ≥16 (see text).	Strength
STRONG CRITERIA		
PS1	Same amino acid change as a previously established pathogenic variant regardless of nucleotide change OR different variant at same nucleotide position as a pathogenic splicing variant, where *in silico* models predict impact equal to or greater than the known pathogenic variant.	Disease-specific
PS2	*De novo* (both maternity and paternity confirmed) observation in a patient with the disease and no family history.	None
PS3	Well-established *in vitro* or *in vivo* functional studies supportive of a damaging effect on the gene or gene product. • Phosphatase activity <50% of wild-type • RNA, mini-gene, or other assay shows impact on splicing	Disease-specific
PS4	Probands with specificity score 4-15.5 (see text) OR The prevalence of the variant in affected individuals is significantly increased compared with the prevalence in controls.	Strength
PM6_Strong	Two probands with presumed *de novo* occurrence (maternity/ paternity not confirmed) with the disease and no family history.	Strength
PP1_Strong	Co-segregation with disease in multiple affected family members, with ≥7 meioses observed across at least two families.	Strength
MODERATE CRITERIA		
PM1	Located in a mutational hot spot and/or critical and well-established functional domain. Defined to include residues in catalytic motifs: 90-94, 123-130, 166-168 (NP_ 000305.3).	Disease-specific
PM2	Present at <0.00001 (0.001%) allele frequency in gnomAD or another large sequenced population. If multiple alleles are present within any subpopulation, allele frequency in that subpopulation must be <0.00002 (0.002%).	Disease-specific

PM3	*For recessive disorders, detected in trans with a pathogenic variant.*	*N/A*
PM4	Protein length changes due to in-frame deletions/insertions in a non-repeat region or stop-loss variants. Applies to in-frame insertions or deletions impacting at least one residue in a catalytic motif (see PM1), protein truncation with disruption starting 3' of c.1121 (NM_000314.6), and variants causing protein extension.	Disease-Specific
PM5	Missense change at an amino acid residue where a different missense change determined to be pathogenic or likely pathogenic has been seen before. In addition, variant being interrogated must have BLOSUM62 score equal to or less than the known variant.	Disease-Specific
PM6	Assumed *de novo,* but without confirmation of paternity and maternity, in proband with the disease and no family history.	None
PS4_Moderate	Probands with specificity score of 2-3.5 (see text).	Strength
PP1_Moderate	Co-segregation with disease in multiple affected family members, with 5 or 6 meioses observed.	Strength
SUPPORTING CRITERIA		
PP1	Co-segregation with disease in multiple affected family members, with 3 or 4 meioses observed.	Disease-Specific
PP2	Missense variant in a gene that has a low rate of benign missense variation and where missense variants are a common mechanism of disease.	None
PP3	Multiple lines of computational evidence support a deleterious effect on the gene or gene product. To be applied only to synonymous or intronic variants where at least 2 out of 3 *in silico* models predict a splicing impact.	Disease-Specific
PP5	*Reputable source recently reports variant as pathogenic but the evidence is not available to the laboratory to perform an independent evaluation*	*N/A*
PS3_ Supporting	Abnormal *in vitro* cellular assay or transgenic model with phenotype different from wild type that does not meet PS3.	Strength; Disease-Specific
PS4_Supporting	Phenotype specific for disease with single genetic etiology. Proband(s) with specificity score of 1-1.5 (see text).	Disease-Specific

BENIGN CRITERIA		
Criteria	**Criteria Description**	**Specification**
STAND ALONE CRITERIA		
BA1	Allele frequency ≥0.01 (1%) in a studied population with ≥2,000 alleles tested and variant present in ≥5 alleles.	Disease-Specific
STRONG CRITERIA		
BS1	Allele frequency from 0.001 (0.1%) up to 0.01 (1%) in a studied population with ≥2,000 alleles tested and variant present in ≥5 alleles.	Disease-Specific

BS2	Observed in the homozygous state in a healthy or PHTS-unaffected individual. One observation if homozygous status confirmed, two if not confirmed. To be applied at supporting evidence level if BS1 is also applied.	Disease-Specific
BS3	Well-established *in vitro* or *in vivo* functional studies shows no damaging effect on protein function. To be applied for missense variants with both lipid phosphatase activity AND results from a second assay appropriate to the protein domain demonstrating no statistically significant difference from wild type. For intronic or synonymous variants, RNA, mini-gene or other splicing assay demonstrates no splicing impact.	Disease-Specific
BS4	Lack of segregation in affected members of two or more families.	Disease-Specific
SUPPORTING CRITERIA		
BP1	*Missense variant in gene where only LOF causes disease*	*N/A*
BP2	Observed *in trans* with a pathogenic or likely pathogenic *PTEN* variant OR at least three observations *in cis* and/or phase unknown with different pathogenic/likely pathogenic *PTEN* variants.	Disease-Specific
BP3	*In-frame deletions/insertions in a repetitive region without a known function*	*N/A*
BP4	Multiple lines of computational evidence suggest no impact on gene or gene product. To be applied only to synonymous or intronic variants where at least 2 out of 3 *in silico* models predict no splicing impact.	Disease-Specific
BP5	Variant found in a case with an alternate molecular basis for disease. Other gene/disorder must be considered highly penetrant AND patient's personal/family history must demonstrate no overlap between other gene and PTEN.	Disease-Specific
BP6	*Reputable source recently reports variant as benign but the evidence is not available to the laboratory to perform an independent evaluation*	*N/A*
BP7	A synonymous (silent) or intronic variant at or beyond +7/-21 for which splicing prediction algorithms predict no impact to the splice consensus sequence nor the creation of a new splice site AND the nucleotide is not highly conserved.	Disease-Specific
BS2_Supporting	Two homozygous observations with no clinical data provided, or meets criteria for BS2 but BS1 is also applied.	Strength; Disease-Specific
BS3_Supporting	*In vitro* or *in vivo* functional study or studies showing no damaging effect on protein function but BS3 not met.	Strength; Disease-Specific
BS4_Supporting	Lack of segregation in affected members of one family.	Strength; Disease-Specific

Key: **Disease-Specific:** Disease-specific modifications based on what is known about PTEN; **Strength:** Increasing or decreasing strength of criteria based on the amount of evidence; **N/A:** not applicable for PTEN; **None:** no changes made to existing criteria definitions.

VERY STRONG EVIDENCE OF PATHOGENICITY

PVS1 Null variant (nonsense, frameshift, canonical +/-1 or 2 splice sites, initiation codon, single or multi-exon deletion) in a gene where loss of function (LOF) is a known mechanism of disease.

PTEN EP Specification: For nonsense or frameshift variants at the 3' end of the gene NOT predicted to result in nonsense-mediated decay, PVS1 may still be applied if the protein is disrupted at or 5' to c.1121 (NM_000314.6). Please see supplementary information in manuscript for evidence supporting this cutoff.

STRONG EVIDENCE OF PATHOGENICITY

PS1 Same amino acid change as a previously established pathogenic variant regardless of nucleotide change.

PTEN EP Specification: PS1 will be applied as described and expanded to include a different nucleotide substitution for an intronic splice site variant if the predicted impact is equal to or greater than the known pathogenic variant per *in silico* splicing tools. Caution should be used when applying this criteria to exonic variants causing aberrant splicing.

PS2 *De novo* (both maternity and paternity confirmed) in a patient with the disease and no family history. Note: Confirmation of paternity only is insufficient. Egg donation, surrogate motherhood, errors in embryo transfer, etc. can contribute to non-maternity.

PS2_Very Strong: Two or more occurrences of PS2 OR two or more occurrences of PM6 AND one occurrence of PS2.

PS3 Well-established *in vitro* or *in vivo* functional studies supportive of a damaging effect on the gene or gene product.

PTEN EP Specification: PS3 may be applied to the following assays:

- *In vitro* or *in vivo* assay demonstrating ≥50% reduction in phosphatase activity compared to wild type control. Phosphatase assays for which criteria may be applied must include a catalytic dead control, such as p.C124S, as well as at least three biological replicates (Myers 1998, Stambolic 1998, Han 2000, Rodriguez-Escudero 2011, Costa 2015, Malek 2017).
- RNA, mini-gene, or other assay demonstrating an impact on splicing.

PS3_Supporting: Abnormal *in vitro* cellular assay or transgenic model with phenotype different from wild-type that does not meet PS3. Examples of *in vitro* cellular assays to be considered for PS3_supporting evidence may include:

- Decreased PTEN or increased pAKT expression (Tan 2011, Spinelli 2015).
- Disruption of protein cellular localization (Lobo 2009, He 2012, Gil 2015).
- Aberrant cellular phenotypes, including defective cell migration, proliferation, and invasion (Costa 2015, Malek 2017).

PS4 Use 1: The prevalence of the variant in affected individuals is significantly increased compared with the prevalence in controls.

PTEN EP Commentary: This criterion is unlikely to be used in this manner for a condition as rare as PHTS. However, if sufficiently powered, a case-control study finding an odds ratio ≥2 for a PHTS component phenotype with p<0.05 and 95% confidence interval with lower limit ≥1.5, this criteria may be applied. However, this criterion may *not* be applied in combination with PP4.

Use 2: Patient's phenotype or family history is highly specific for a disease with a single genetic etiology.

PTEN EP Specifications: This criterion may not be applied if BS1 applies. Phenotype specificity scores are added across independent probands and calculated as follows:

- Adults:
  - 1 point per proband with Cleveland Clinic (CC) score ≥30 (Tan 2011)
  - 0.5 points per proband with CC score of 25-29.
- Children:
  - 1 point per proband with pediatric phenotype score ≥5 (please see supplementary information in manuscript for scoring rubric).
  - 0.5 points per proband with pediatric phenotype score of 4, but autism/developmental delay/intellectual disability may not contribute to the score.

PS4_Very Strong: Probands with specificity score ≥16.

PS4: Probands with specificity score of 4-15.5.

PS4_Moderate: Probands with specificity score of 2-3.5.

PS4_Supporting: Proband(s) with specificity score of 1-1.5.

MODERATE EVIDENCE OF PATHOGENICITY

PM1 Located in a mutational hot spot and/or critical and well-established functional domain (e.g. active site of an enzyme) without benign variation.

PTEN EP Specification: Defined to include residues in one of PTEN's catalytic motifs, which include the WPD loop (residues 90-94), P-loop (also described as phosphatase core, residues 123-130), and the TI-loop (residues 166-168) (NP_ 000305.3) (Lee 1999).

PM2 Absent from controls (or at extremely low frequency if recessive) in Exome Sequencing Project, 1000 Genomes or ExAC.

PTEN EP Specification: Criteria may be applied if a variant is present at <0.00001 (0.001%) allele frequency in gnomAD or another large sequenced population. If multiple alleles are present within a subpopulation, allele frequency in that subpopulation must be <0.00002 (0.002%). Please see supplementary information in manuscript supporting application of PM2 for ultra-rare alleles.

PM3 For recessive disorders, detected *in trans* with a pathogenic variant.

PTEN EP Commentary: This rule is not applicable to PTEN.

PM4 Protein length changes due to in-frame deletions/insertions in a non-repeat region or stop-loss variants.

PTEN EP Specification: For in-frame insertions or deletions, criteria may apply only if the variant impacts at least one residue in one of the catalytic motifs specified in the PM1 criteria. Criteria will also apply for variants resulting in truncation 3' to c.1121 (NM_000314.6) or variants resulting in protein extension.

PM5 Novel missense change at an amino acid residue where a different missense change determined to be pathogenic has been seen before.

PTEN EP Specifications:

- This rule may be applied when the known variant is likely pathogenic unless applying would lead to a higher (pathogenic) classification for the variant being assessed.
- The variant in question need not be novel but must have a BLOSUM62 (Henikoff 1992) score equal to or less than the known variant.

PM6 Assumed de novo, but without confirmation of paternity and maternity in a patient with the disease and no family history.

PM6_Very Strong: Four or more occurrences of PM6 OR two occurrences of PM6 AND one occurrence of PS2.

PM6_Strong: Two occurrences of PM6.

SUPPORTING EVIDENCE OF PATHOGENICITY

PP1 Co-segregation with disease in multiple affected family members in a gene definitively known to cause the disease.

PTEN EP Specification: Requires 3 or 4 meioses in order to apply.

PP1_Strong: At least 7 meioses required across at least two families.

PP1_Moderate: Requires 5 or 6 meioses in order to apply.

PP2 Missense variant in a gene that has a low rate of benign missense variation and where missense variants are a common mechanism of disease.

PP3 Multiple lines of computational evidence support a deleterious effect on the gene or gene product (conservation, evolutionary, splicing impact, etc).

PTEN EP Specification: To be applied only to synonymous or intronic variants where at least 2 out of 3 in silico models predict a splicing impact. Not to be applied for variants which may impact the intron 1 splice donor or acceptor sites, and to be used cautiously for variants which may impact the intron 6 splice acceptor.

PTEN EP Commentary: Given the lack of known benign or likely benign *PTEN* missense variants, the Expert Panel was unable to test the accuracy of *in silico* predictors to be used as evidence to apply BP4 or PP3 for *PTEN* missense variants. While investigating potential *in silico* tools, the Expert Panel also came to find that some algorithm predictions were highly sensitive to sequence alignment, further limiting confidence in these tools. Should the Expert Panel classify several missense variants as benign or likely benign, another attempt will be made to validate *in silico* tools to apply PP3/BP4 for missense variants. Please see supplementary information in manuscript detailing validation of splicing *in silico* tools and challenges presented by the specified donor/acceptor sites.

PP4 Patient's phenotype or family history is highly specific for a disease with a single genetic etiology.

PTEN EP Commentary: Phenotype specificity has been incorporated into the rule specifications for PS4 Use 2.

PP5 Reputable source recently reports variant as pathogenic but the evidence is not available to the laboratory to perform an independent evaluation.

PTEN EP Commentary: This rule is not applicable to PTEN.

STAND ALONE EVIDENCE OF BENIGN IMPACT

BA1 Allele frequency is above 5% in Exome Sequencing Project, 1000 Genomes, or ExAC.

PTEN EP Specification: To be applied for variants with allele frequency ≥0.01 (≥1%) in a studied population with ≥2,000 alleles tested and variant present in ≥5 alleles. Please see supplementary information in manuscript for data supporting this lowered allele frequency threshold.

STRONG EVIDENCE OF BENIGN IMPACT

BS1 Allele frequency is greater than expected for disorder.

PTEN EP Specification: To be applied for variants with allele frequency of 0.001 up to 0.01 (0.1% up to 1%) in a studied population with ≥2,000 alleles tested and variant present in ≥5 alleles. Please see supplementary information in manuscript for data supporting this lowered allele frequency threshold.

BS2 Observed in a healthy adult individual for a recessive (homozygous), dominant (heterozygous), or X-linked (hemizygous) disorder with full penetrance expected at an early age.

PTEN EP Specifications: Variant must be observed in the homozygous state in a healthy or PHTS-unaffected individual. Two independent observations are required if the homozygous status is not confirmed via parental testing. If BS1 is also applied, this criteria will be applied at the supporting evidence level to avoid a variant reaching benign status solely based on homozygous occurrences due to high population frequency (BS1+BS2).

BS2_Supporting: Two homozygous observations with no clinical data provided, or meets criteria for BS2 but BS1 is also applied.

BS3 Well-established *in vitro* or *in vivo* functional studies show no damaging effect on protein function or splicing.

PTEN EP Specifications: BS3 may be applied to the following assays:

- For missense variants: Lipid phosphatase activity comparable to wild type in addition to a second assay appropriate to the protein domain demonstrating no statistically significant difference from wild type. Phosphatase assays for which criteria may be applied must include a catalytic dead control, such as p.C124S (NP_ 000305.3), as well as at least three biological replicates (Myers 1998, Stambolic 1998, Han 2000, Rodriguez-Escudero 2011, Costa 2015, Malek 2017). Examples of second assays may include:
  - Decreased PTEN or increased pAKT expression (Tan 2011, Spinelli 2015).
  - Disruption of protein cellular localization (Lobo 2009, He 2012, Gil 2015).
  - Aberrant cellular phenotypes, including defective cell migration, proliferation, and invasion (Costa 2015, Malek 2017).
- For intronic or synonymous variants: RNA, mini-gene, or other assay demonstrate no impact on splicing.

BS3_Supporting: *In vitro* or *in vivo* functional study or studies showing no damaging effect on protein function but BS3 not met.

BS4 Lack of segregation in affected members of a family.

PTEN EP Specification: Two or more families are require for strong evidence level.

BS4_Supporting: Lack of segregation in one family.

SUPPORTING EVIDENCE FOR BENIGN IMPACT

BP1 Missense variant in a gene for which primarily truncating variants are known to cause disease.

PTEN EP Commentary: This rule is not applicable to PTEN.

BP2 Observed *in trans* with a pathogenic variant for a fully penetrant dominant gene/disorder; or observed in cis with a pathogenic variant in any inheritance pattern.

PTEN EP Specifications: The other variant may be either pathogenic or likely pathogenic. This rule may also be applied for at least three observations of the variant *in cis* or unknown phase with different pathogenic or likely pathogenic *PTEN* variants.

BP3 In-frame deletions/insertions in a repetitive region without a known function.

PTEN EP Commentary: This rule is not applicable to PTEN.

BP4 Multiple lines of computational evidence suggest no impact on gene or gene product (conservation, evolutionary, splicing impact, etc.).

PTEN EP Specification: To be applied only to synonymous or intronic variants where at least 2 out of 3 *in silico* models predict no splicing impact. Not to be applied for variants which may impact the intron 1 splice donor or acceptor sites, and to be used cautiously for variants which may impact the intron 6 splice acceptor.

PTEN EP Commentary: Please see PP3 commentary.

BP5 Variant found in a case with an alternate molecular basis for disease.

PTEN EP Specifications: At least two such cases are required for criteria to apply. In addition, the other gene/disorder must be considered highly penetrant AND the patient's personal/family history must demonstrate no overlap between the other gene and *PTEN*.

BP6 Reputable source recently reports variant as benign but the evidence is not available to the laboratory to perform an independent evaluation.

PTEN EP Commentary: This rule is not applicable to PTEN.

BP7 A synonymous (silent) variant for which splicing prediction algorithms predict no impact to the splice consensus sequence nor the creation of a new splice site AND the nucleotide is not highly conserved.

PTEN EP Specification: Intronic variants must be positioned at or beyond +7/-21. Nucleotide may be defined as "not conserved" with PhastCons score <1 and PhyloP score <0.1.

RULES FOR COMBINING CRITERIA FOR CLASSIFICATION

No changes from the rules provided in Richards et al. (2015). Variants will be defined as having contradictory evidence when criteria for both pathogenic/likely pathogenic and benign/likely benign classification are met. As an example, a variant with PM1, PM2, PM6, BP4, and BP7 applied would meet both likely pathogenic and likely benign criteria and thus be considered contradictory, leading to an expert panel classification of VUS. However, a variant with PM2, BP4, and BP7 applied would be considered likely benign.

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